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Supplemental Material

Hepatic Lipid Accumulation and Nrf2 Expression following Perinatal and Peripubertal Exposure to Bisphenol A (BPA) in a Mouse Model of Nonalcoholic Liver Disease

Prajakta C. Shimpi, Vijay R. More, Maneesha Paranjpe, Ajay C. Donepudi, Jaclyn M. Goodrich, Dana C. Dolinoy, Beverly Rubin, and Angela L. Slitt

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Supplementary Table S1: Primer sequences for quantitative RT-PCR

Mouse primers:

Gene	Forward	Reverse
Srebp-1c	GCAGCCACCATCTAGCCTG	CAGCAGTGAGTCTGCCTTGAT
Ppar-γ	CTCTGTTTTATGCTGTTATGGGTGA	GGTCAACAGGAGAATCTCCCAG
Fas	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
Acc-1	GATGAACCATCTCCGTTGGC	GACCCAATTATGAATCGGGAGTG
Scd-1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
Gpat	ACAGTTGGCACAATAGACGTTT	CCTTCCATTTCAGTGTTGCAGA
Nrf2	TCTTGGAGTAAGTCGAGAAGTGT	GTTGAAACTGAGCGAAAAAGGC
Keap1	CTGCCCAATTCATGGCTCACA	CTTAGGGTGGATGCCTTCGAT
Nqo1	AGGATGGGAGGTACTCGAATC	TGCTAGAGATGACTCGGAAGG
Gclc	GGGGTGACGAGGTGGAGTA	GTTGGGGTTTGTCCTCTCCC
B2M	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC
ApoB	AAGCACCTCCGAAAGTACGTG	CTCCAGCTCTACCTTACAGTTGA
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Fabppm	GGACCTCCAGATCCCATCCT	GGTTTTCCGTTATCATCCCGGTA
Fatp2	TCCTCCAAGATGTGCGGTACT	TAGGTGAGCGTCTCGTCTCG
Fatp5	CTACGCTGGCTGCATATAGATG	CCACAAAGGTCTCTGGAGGAT
Cd36	CGCTTTCTGCGTATCGTCTG	GATGCACGGGATCGTGTCT
G6Pase	CGACTCGCTATCTCCAAGTGA	GTTGAACCAGTCTCCGACCA
Pepck	CTGCATAACGGTCTGGACTTC	CAGCAACTGCCCGTACTCC
Pgc-1α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
Ppar-α	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
Irs-1	CTCTACACCCGAGACGAACAC	TGGGCCTTTGCCCGATTATG
Irs-2	CTGCGTCCTCTCCCAAAGTG	GGGGTCATGGGCATGTAGC
Akt-1	ATGAACGACGTAGCCATTGTG	TTGTAGCCAATAAAGGTGCCAT
Akt-2	CCACGACCCAACACCTTGT	GATAGCCCGCATCCACTCTTC
Cyp4a10	AGAACTTCCCAAGTGCCTTTC	GCAAACCATACCCATTAGCCTTT
Glut2	TCAGAAGACAAGATCACCGGA	GCTGGTGTGACTGTAAGTGGG
TNFα	TCTCATGCACCACCATCAAGGACT	ACCACTCTCCCTTTGCAGAA TCA
IL-6	ATCCAGTTGCCTTCTTGGGACTGA	TAAGCCTCCGACTTGTGAAGTGGT

IL1- β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Mcp-1	GGCTCAGCCAGATGCAGTT	GCTGCTGGTGATCCTCTTGT

Human Primers

Gene	**Forward**	**Reverse**
Srebp-1c	GCGGAGCCATGGATTGCAC	CTCTTCCTTGATACCAGGCCC
Fas	AGCTGCCAGAGTCGGAGAAC	TGTAGCCCACGAGTGTCTCG
Acc-1	TCGCTTTGGGGGAAATAAAGTG	ACCACCTACGGATAGACCGC
Scd	CTTCTTGCGATACACTCTGG	TGAATGTTCTTGTCGTAGGG
Nrf2	GCGACGGAAAGAGTATGAC	GTTGGCAGATCCACTGGTTT
Nqo-1	TCCCCCTGCAGTGGTTTGGAGT	ACTGCCTTCTTACTCCGGAAGGGT
Gclc	GTTCTTGAAACTCTGCAAGAGAAG	ATGGAGATGGTGTATTCTTGTCC
Gapdh	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA

Supplementary Table S2: List of primary and secondary antibodies for western blot and chromatin immunoprecipitation assay

Antibody name	Primary/ Secondary	Protein fraction	Application	Source
Gapdh/ β-actin	Primary	Total, Membrane	WB	Cell Signaling Tech
Acc-1	Primary	Total	WB	Cell Signaling Tech
pAcc-1	Primary	Total	WB	Cell Signaling Tech
Ppar-γ	Primary	Total	WB	Cell Signaling Tech
Srebp1c	Primary	Total	WB	Active Motif
			ChIP	Active Motif
p-Srebp1c		Total	WB	Cell Signaling Tech
Nrf2	Primary	Nuclear	WB	Gift from Dr. Schmidt's lab
			ChIP	Cell Signaling Tech
Gclc	Primary	Total	WB	Abcam
Anti-mouse	Secondary	-	WB	Sigma Aldrich
Anti-Rabbit	Secondary	-	WB	Sigma Aldrich
Anti-rat	Secondary	-	WB	Sigma Aldrich, St Louis, MO

Supplementary Table S3: Primer sequences for ChIP

Promoter	Predicted ARE (Considering Coding start site as +1)	Forward	Reverse
Srebp1c	-120 to -130	TAGGCGAGCTGTCAGGAT	TCTCGGCCAGTGTCTGT

Supplementary Table S4: Primer sequences for methylated DNA immunoprecipitation (MeDIP)

Promoter	Location (coding start site +1)	Number of CpG sites	Forward	Reverse
Srebp1c	-231 to -347	2	CAGCAAGACTAGGAAGTGAGTT	GGCCTTGGCTTCTTCTGTAT
Srebp1c	-1325 to -1456	2	CTGGGACAGGCTATTTGAGTT	GGCTTCACCAGGACACATT
Fas	-306 to -472	5	TGTTCCCTCTGGGTCCTAA	CAGTTGCCTACTGGATGCT
Fas	- 654 to -832	9	CAGCTTGGAGAAGGCAGATG	CTGGTCGAACACTTTGTAACCT
Nrf2	-1059 to-1169	4	GGTCACCACAACACGAACTA	GACTCTCAGGGTTCCTTTACAC

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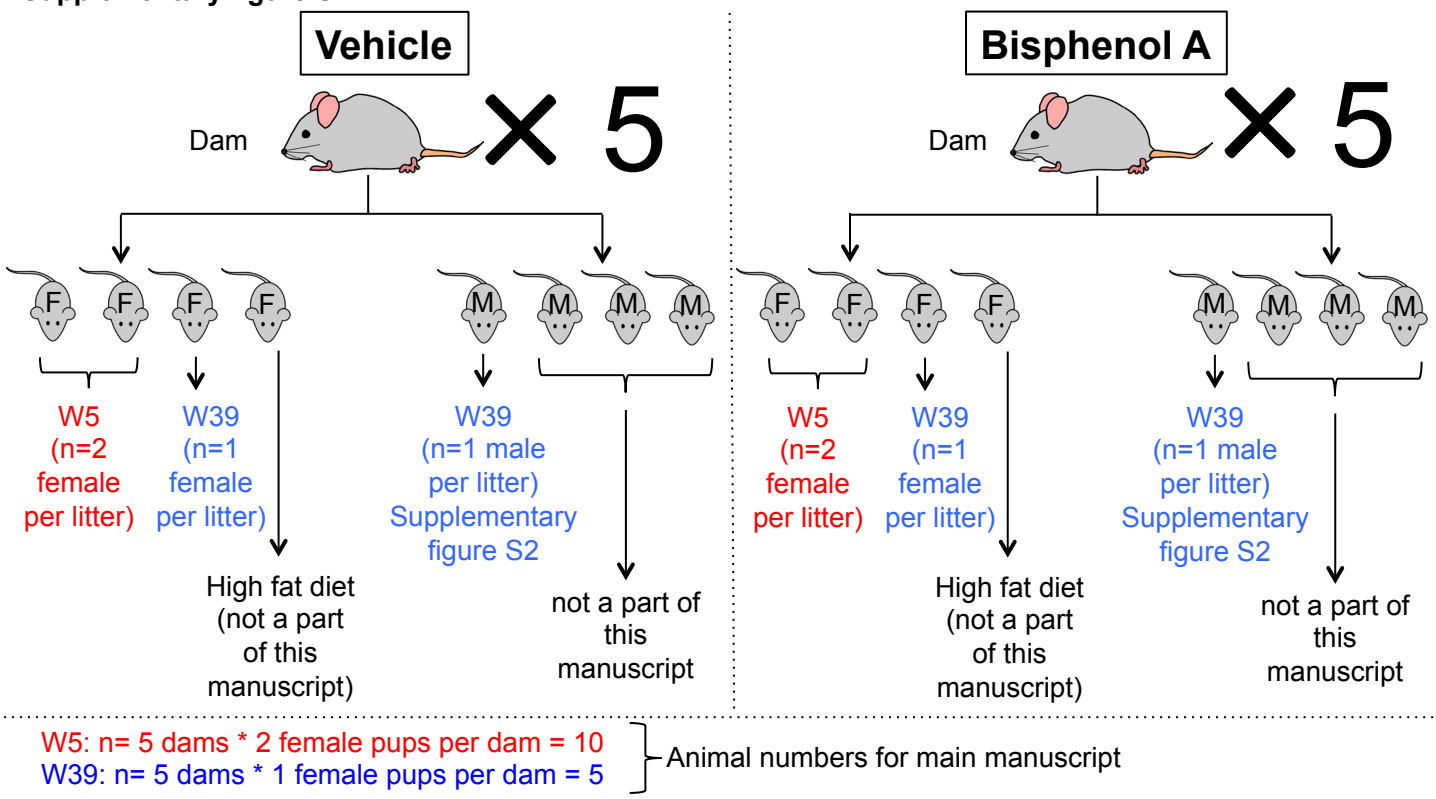
Supplementary Table S5: PCR conditions and primer sequences for Pyro-Sequencing

Primer/sequence to analyze	***Nrf2* Assay**
Forward primer	GTAGTTAAAGAAGTATGTTTGGGAATGA
Reverse primer	Biotin-TATAATCTCATAAAACCCCACCTCTC
Sequencing primer (5' to 3')	ATAATAAGAATTATATTAAAGGG
Amplicon length	318
Annealing Temperature	58
Cycles	45

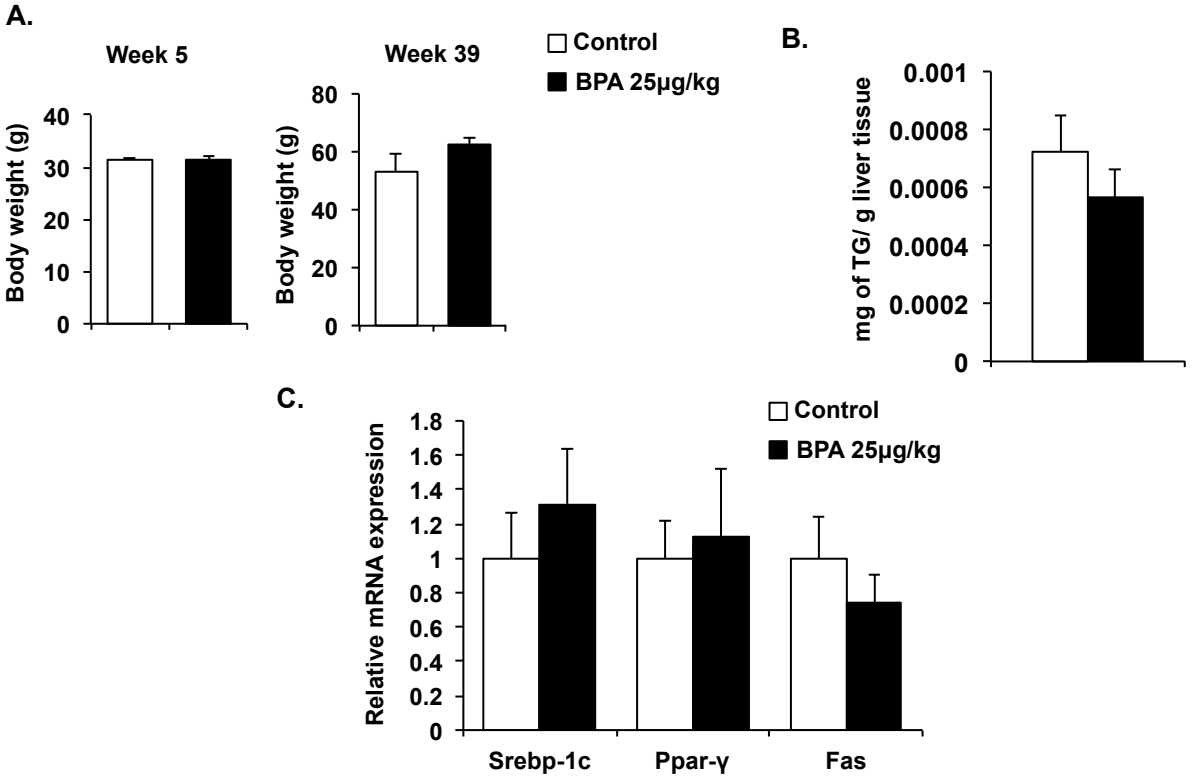
Supplementary figure S1



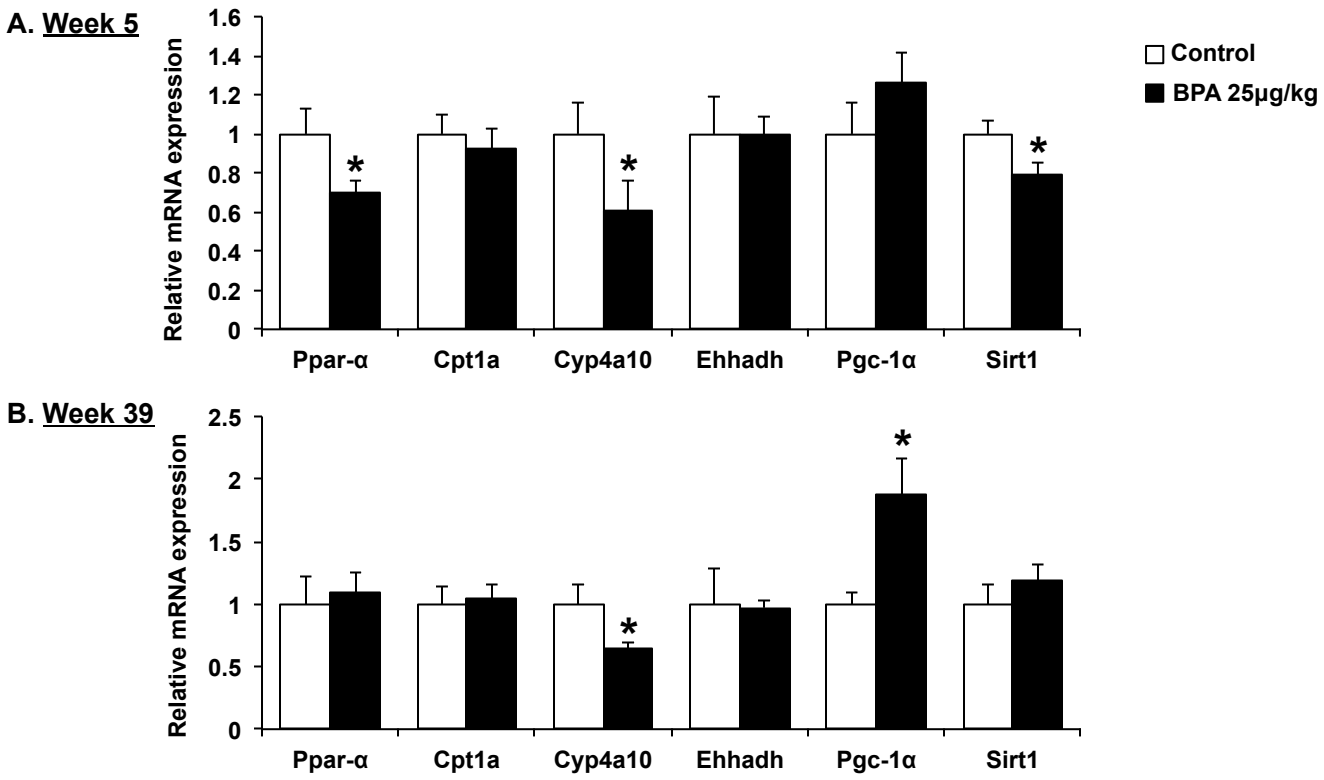
W5: n= 5 dams * 2 female pups per dam = 10
W39: n= 5 dams * 1 female pups per dam = 5
} Animal numbers for main manuscript

<u>Supplementary figure S1:</u> Study design for perinatal peripubertal (PNPP) BPA exposure and explanation for animal number per group. The study included n=5 dams per treatment group (Vehicle or BPA) and 1 litter per dam. Pups were culled to 8 pups per litter (4 males and 4 females) the day after birth (PND2). Two female mice were chosen randomly per dam for W5 studies (n=10), and one female per dam was chosen for W39 studies (n=5). Another female pup was subjected to high fat diet study. One male pup for dam was also studied at W39 (n=5)

Supplementary figure S2

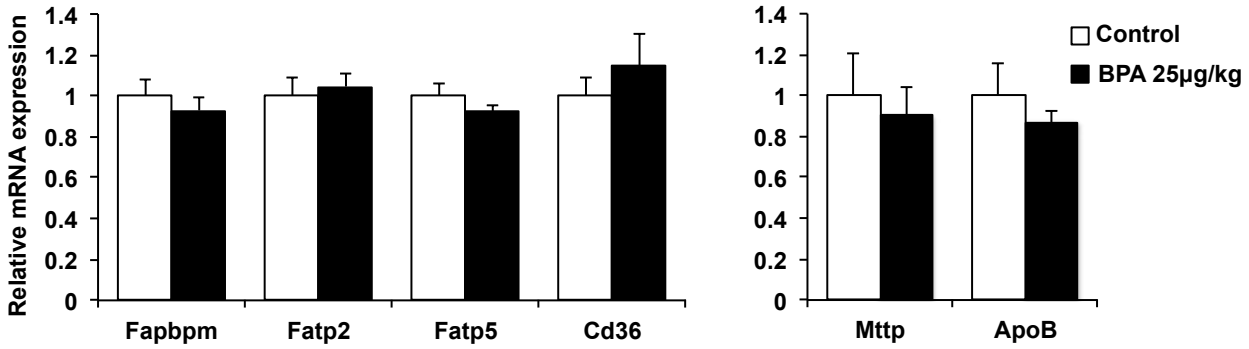


Supplementary figure S2: Effect of perinatal peripubertal (PNPP) BPA exposure on hepatic triglyceride (TG) and mRNA expression in male offspring. A. Body weight (W5 and W39) at the time of necropsy. **B.** TG quantification (W39). **C.** Gene expression of lipogenic targets (W39). Raw data was normalized to respective control expression, and statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). Hepatic TG and lipogenic gene expression remained unchanged after PNPP BPA exposure in male mice.

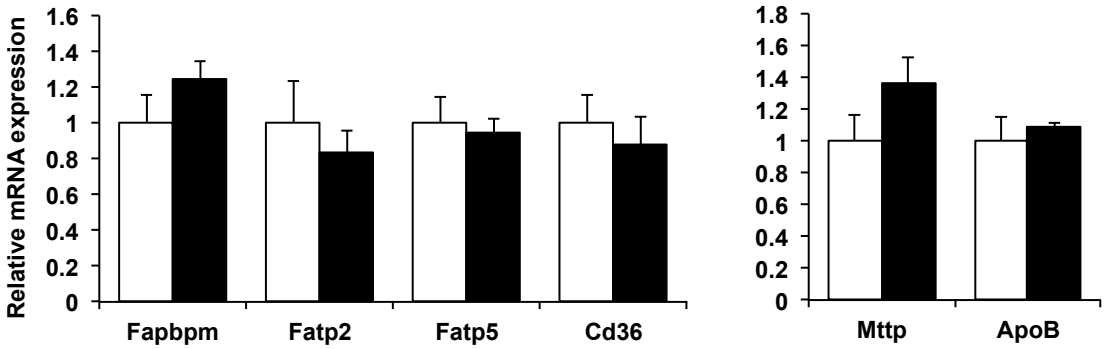


Supplementary figure S3: Evaluation of perinatal peripubertal (PNPP) exposure to BPA mRNA expression of β-oxidation targets and lipolytic genes in livers of female CD-1 mice (A. Week 5; B. Week 39). Messenger RNA was converted to cDNA and subsequently quantified using real time polymerase chain reaction (RT-PCR) using primers specific for peroxisome proliferator-activated receptor alpha (Ppar-α), cytochrome P450 4a10 (Cyp4a10), carnitine palmitoyltransferase 1a (Cpt1a), Enoyl-CoA, 3-hydroxyacyl CoA Dehydrogenase, a proximal L-bifunctional protein (Ehhdh), proliferator-activated receptor gamma coactivator-1 alpha (Pgc1-α), and sirtuin1 (Sirt1). Raw data was normalized to respective control expression, and statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). PNPP BPA exposure suppresses expression of few of lipolytic genes at W5, but effects were not consistent in W39.

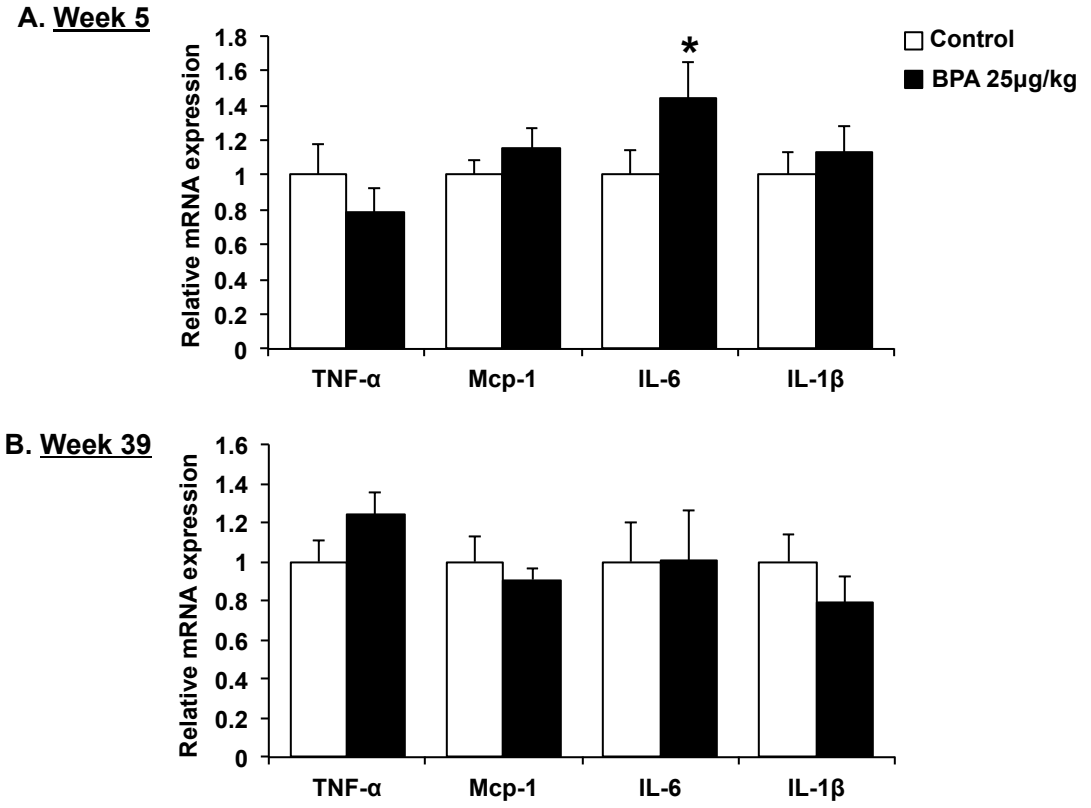
A. Week 5



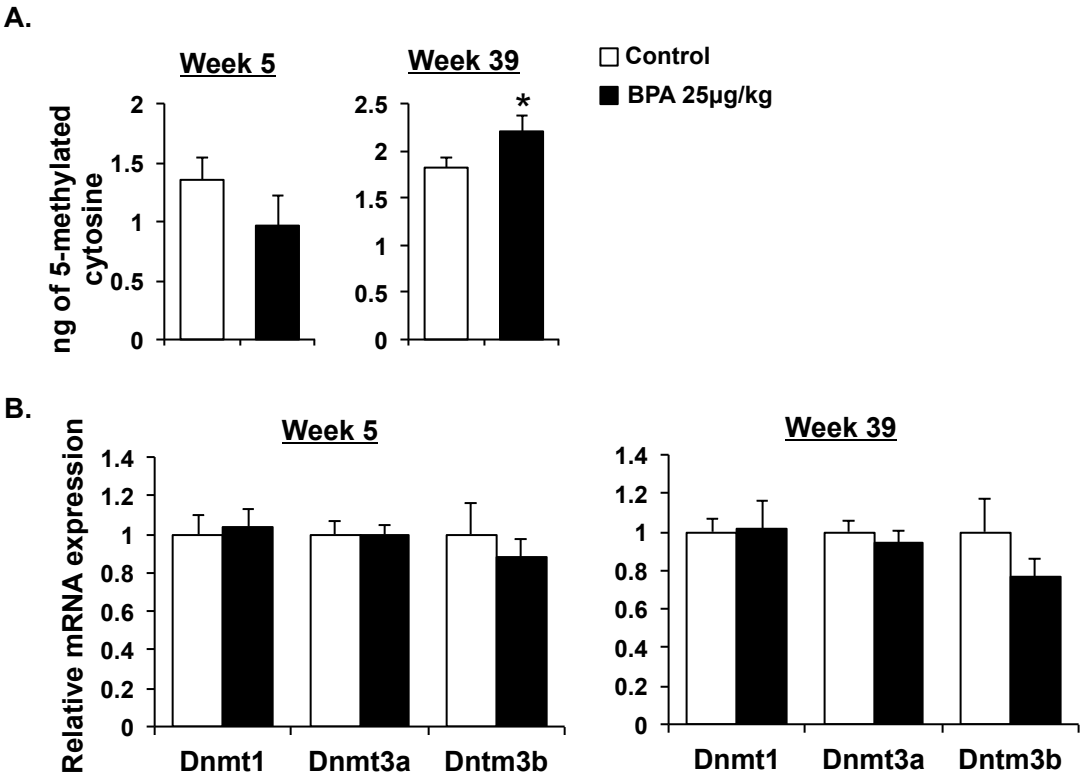
B. Week 39



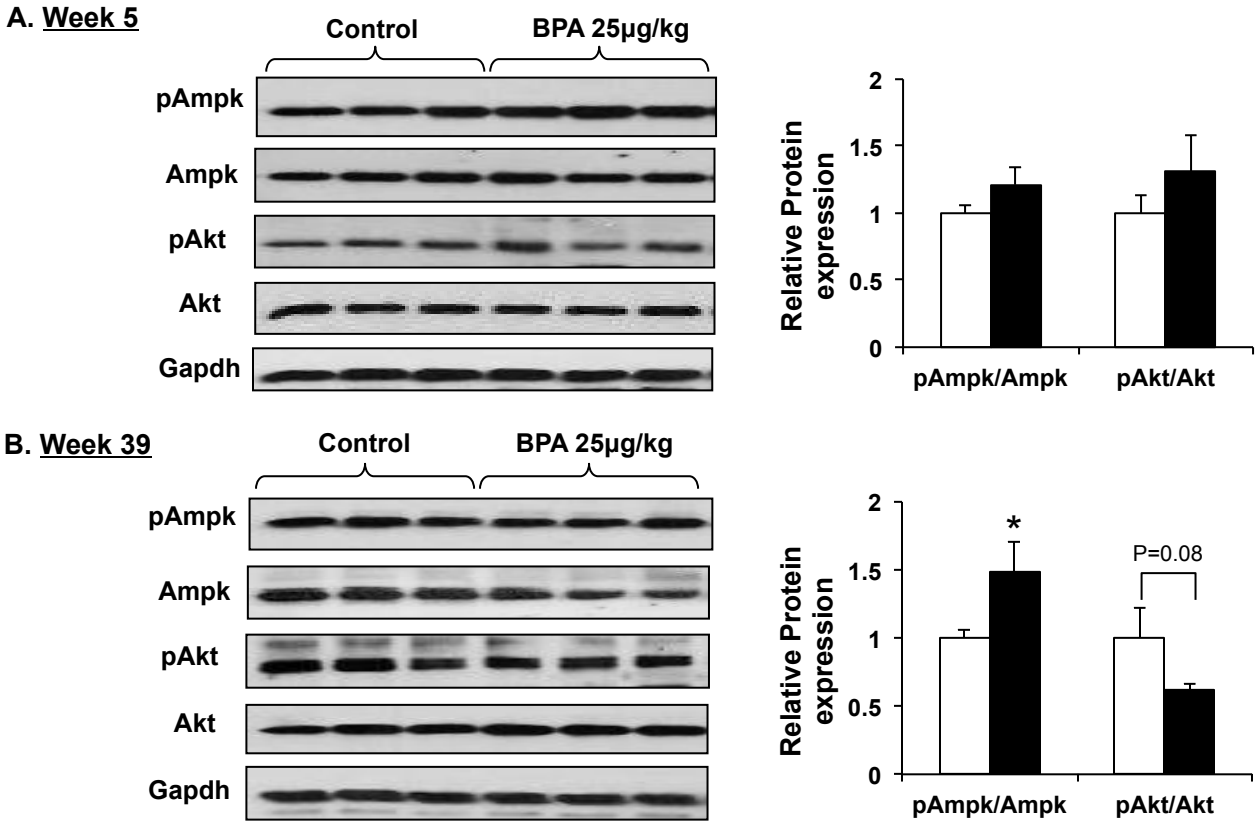
Supplementary figure S4: Effect of Perinatal peripubertal (PNPP) exposure to BPA hepatic fatty acid uptake and export pathway in livers of female CD-1 mice (A. Week 5; B. Week 39). mRNA was quantified by RT-PCR using primers specific for fatty acid binding protein (Fabp), fatty acid transport protein (Fatp2 and Fatp5) and fatty acid translocase (Fat/Cd36), microsomal triglyceride transfer protein (MttP), and apolipoprotein B (ApoB). Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). PNPP BPA exposure neither changed fatty acid uptake nor export both in W5 and W39 female livers.



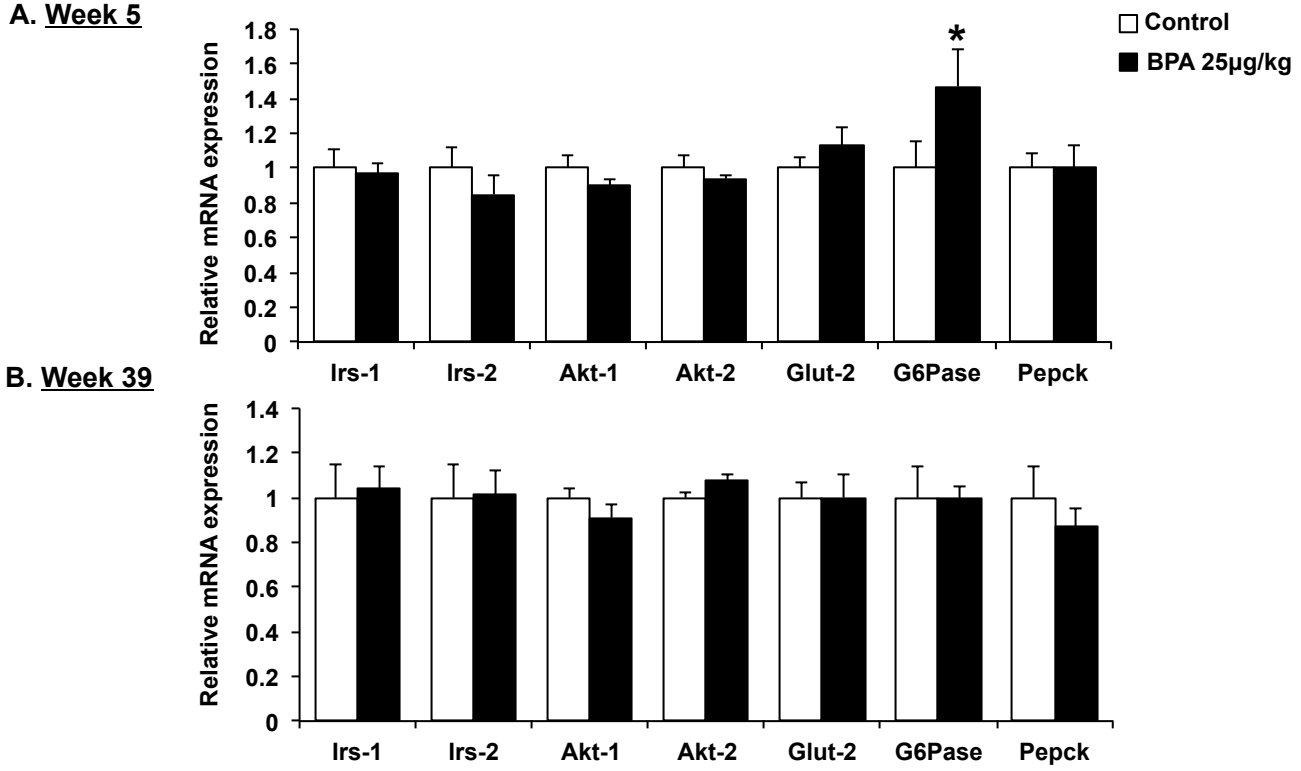
Supplementary figure S5: Evaluation of proinflammatory cytokine and pathway target mRNA expression in livers of female CD-1 mice after perinatal peripubertal (PNPP) exposure to BPA (A. Week 5; B. Week 39). Messenger RNA was converted to cDNA and subsequently quantified using real time polymerase chain reaction (RT-PCR) using primers specific for tumor necrosis factor alpha (Tnf-α), monocyte chemoattractant protein (Mcp1), interleukin-6 (Il-6), Il-1β. Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). Inflammation and insulin signaling targets remained largely unaltered with PNPP BPA exposure, except Il-6 and G6pase which were induced only in W5.



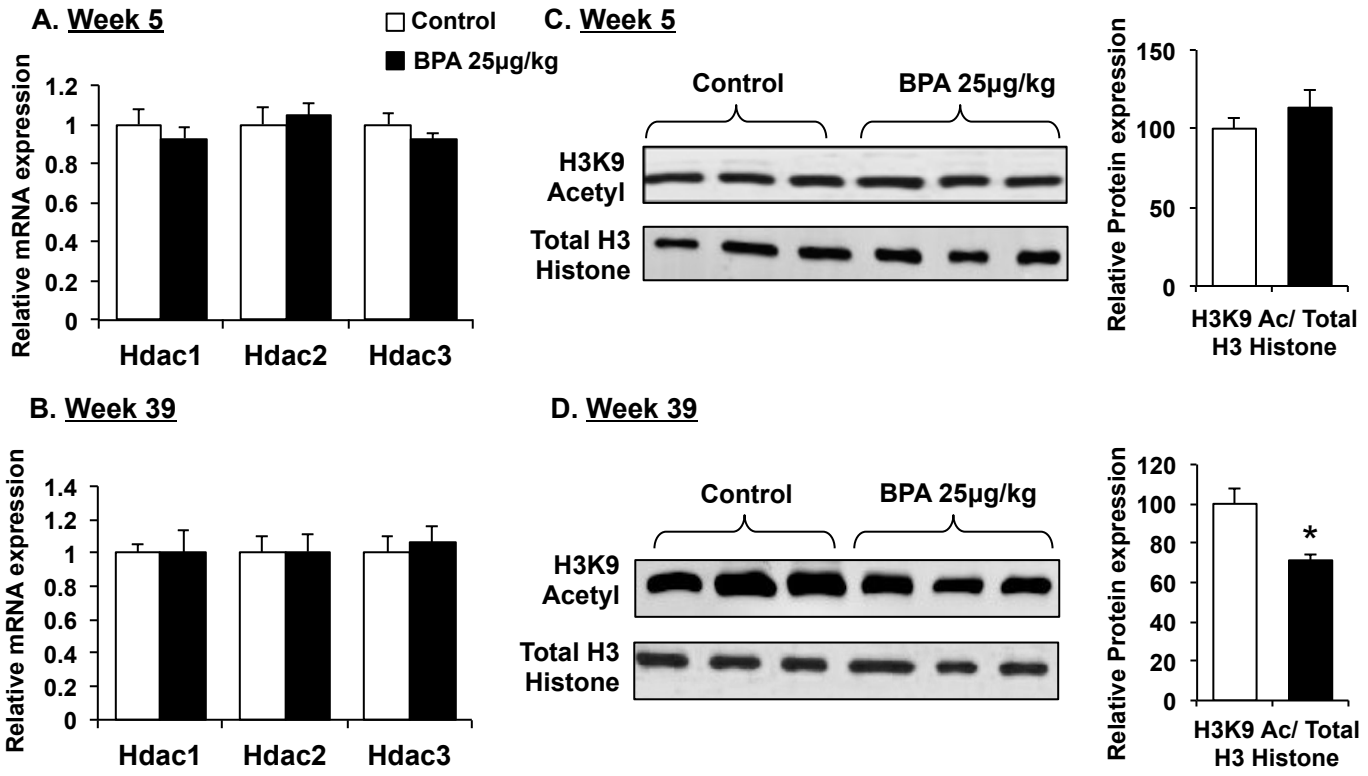
Supplementary figure S6: Effect on global methylation after PNPP exposure to BPA in livers of female CD-1 mice at W5 and W39 age. **A**. Amount of methylated DNA was assessed by quantifying 5-methyalcytosine content using MethylFlash™ Methylated DNA Quantification Kit (Epigentek, Farmingdale NY). PNPP BPA exposure did not affect global DNA methylation in W5, but increase in W39 female livers. **B.** mRNA expression of DNA methyltransferase (Dnmt1, 3a and 3b) enzyme was quantified by RT-PCR using specific primers. Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). PNPP BPA exposure doesn't change Dnmt expression in both W5 and W39.



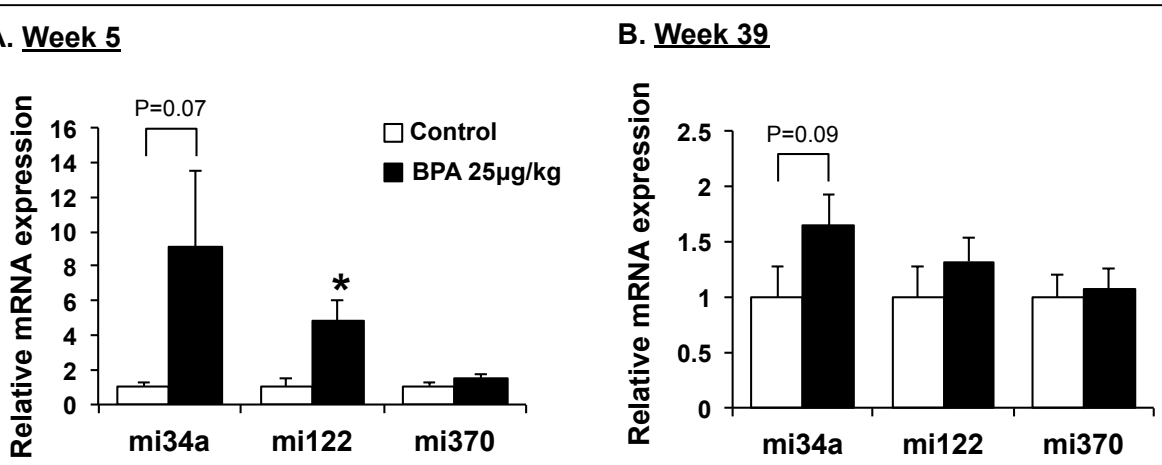
Supplementary figure S7: Ampk and Akt phosphorylation in livers of female CD-1 mice after PNPP exposure to BPA (A. Week 5; B. Week 39). Relative quantification of Ampk and Akt phosphorylation was determined by western blot. The blots were quantified by ImageJ and graphs represent ratio of phosphorylated protein to the total protein expression. Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). In W5 female livers pAmpk/Ampk ratio remains same upon BPA exposure and in W39 female PNPP BPA exposure increased pAMP/Ampk ratio. In both ages pAkt/Akt ratio remained unchanged with BPA expsorue.



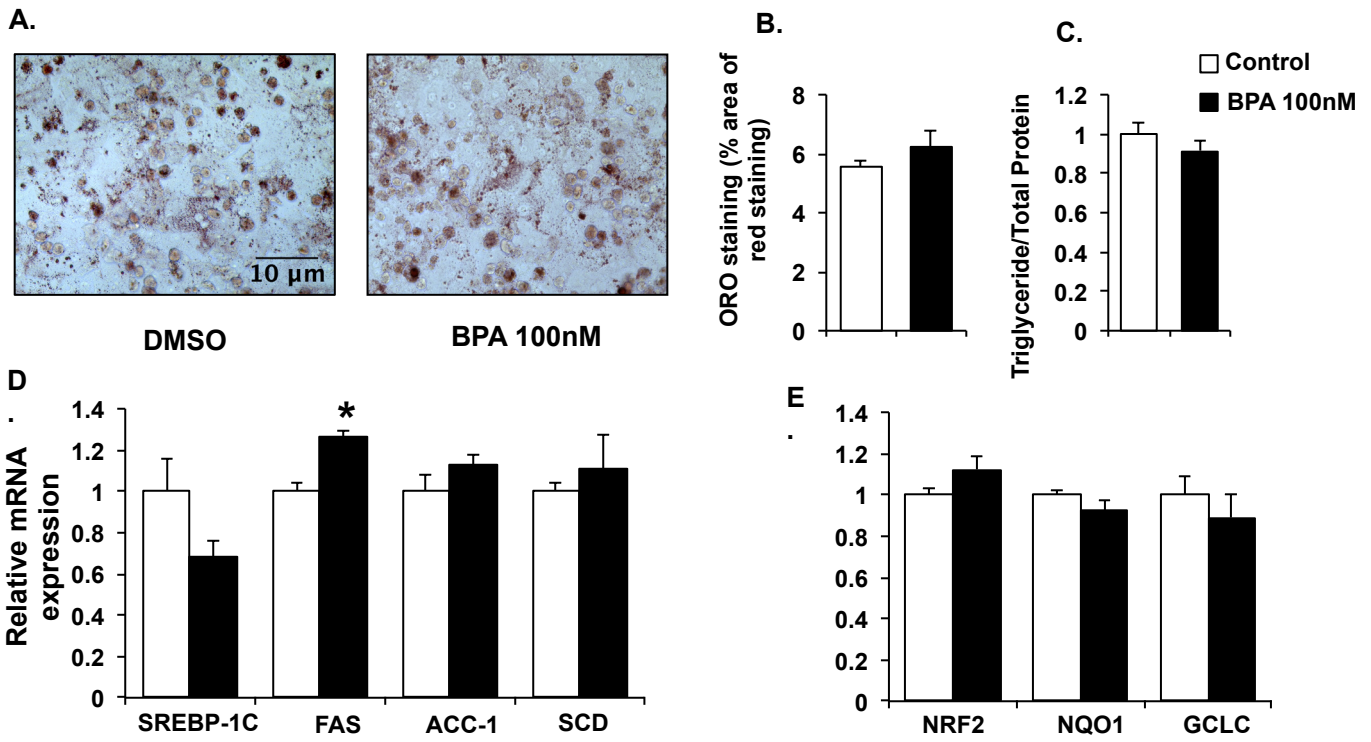
Supplementary figure S8: Evaluation of insulin pathway target mRNA expression in livers of female CD-1 mice after perinatal peripubertal (PNPP) exposure to BPA (A. Week 5; B. Week 39). Messenger RNA was converted to cDNA and subsequently quantified using real time polymerase chain reaction (RT-PCR) using primers specific for insulin receptor substrate 1 (Irs1), Irs2, Akt1, Akt2, glucose transporter 2 (Glut2), glucose-6-phosphatase (G6pase) and phosphoenol pyruvate carboxykinase (Pepck). Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). Inflammation and insulin signaling targets remained largely unaltered with PNPP BPA exposure, except Il-6 and G6pase, which were induced only in W5.



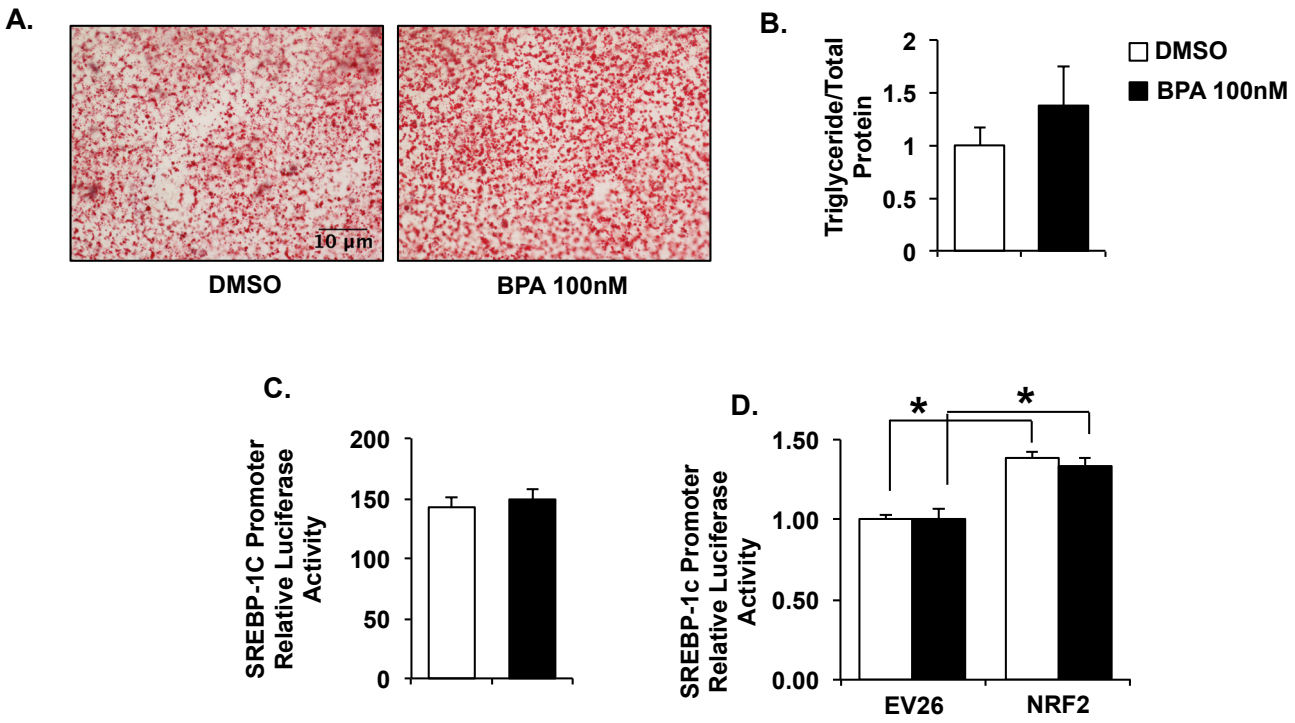
Supplementary figure S9: Effect PNPP exposure of BPA on histone acetylation in livers of female CD-1 mice at W5 and W39 age. A and B. Expression of histone deacetylase (Hdac1, Hdac2, Hdac3) was measured by RT-PCR using specific primers**.** PNPP BPA exposure doesn't alter Hdac expression at both ages (W5 and W39). **C and D.** Relative levels of histone H3 acetylation were measured by western blot analysis of acetylated H3 histone and total H3 histone and calculation the ratio of acetyl-H3K9 to total H3 of blot quantification. Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). Histone acetylation level was not different between control and BPA exposed W5 female liver, however W39 showed less expression of H3K9 with BPA exposure.



Supplementary figure S10: Effect PNPP exposure of BPA on hepatic miRNA in female CD-1 mice (A. Week 5; B. Week 39). The RNA fraction enriched for small RNAs was isolated from female livers exposed PNPP BPA. miR-34a, miR-122, miR-370 expression was quantified by RT-PCR and normalized to U-6 housekeeping expression. Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). Both W5 and W39 livers demonstrates no change in miRNA expression upon BPA exposure, except miR-122 expression was significantly repressed in W5.



Supplementary figure S11: In-vitro exposure of BPA on cultured primary human hepatocyte. **A.** Representative image of Oil Red O staining of primary human hepatocyte treated with BPA (100nM) for 72 hrs along with vehicle (DMSO 0.1%). **B.** Relative quantification of Oil Red O Stain. **C.** Lipids were extracted from primary human hepatocyte after 72hrs of DMSO (0.1%) or BPA (100nM) and treatment by using chloroform/methanol mixture, and triglyceride (TG) content was determined spectrophotometrically. Relative triglyceride content was displayed. Both ORO and TG quantification shows direct BPA exposure does not increase lipid accumulation in primary human hepatocytes. **D and E.** Total RNA was extracted from human hepatocyte pretreated with DMSO or BPA (100nM) for 72 hrs and lipogenic and Nrf2 mRNA level was measured by RT-PCR. . Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). Expression of lipogenic and Nrf2 target genes largely remained unchanged after BPA treatment, except Fas.



<u>Supplementary figure S12:</u> Lipid accumulation and *Srebp-1c* transcriptional activity in HepG2 cells exposed to BPA. Lipid content of HepG2 cells was measured after 48 hrs treatments of DMSO (0.1%) or BPA (100nM) by **A.** Oil Red O staining **B.** Triglyceride quantification. **C.** HepG2 cells were transfected with a *hSREBP-1C* luciferase promoter, followed by BPA exposure and luciferase reporter assay **D.** HeG2 cells were co-transfected with either human NRF2 cDNA plasmid or empty vector (EV26). Cells were exposed to BPA and luciferase assay was conducted. Statistical differences between the groups were analyzed by student's t-test. Asterisk (*) represents significant difference in expression between BPA treated and control animals ($p \leq 0.05$). BPA treatment doesn't modify *hSREBP-1c* promoter luciferase activity as well as doesn't augment regulation of NRF2 on transactivation of *hSREBP-1C* promoter.